

**UTILITY
PATENT APPLICATION
TRANSMITTAL**

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Attorney Docket No.

0010-1057-0

First Inventor or Application Identifier

Yoshihiro USUDA, et al.

Title

METHOD FOR PRODUCING L-METHIONINE BY FERMENTATION

11/16/99
09/441055
JC675 U.S. PTO

APPLICATION ELEMENTS

See MPEP chapter 600 concerning utility patent application contents

ADDRESS TO:

Assistant Commissioner for Patents
Box Patent Application
Washington, DC 20231

1. ☒ Fee Transmittal Form (e.g. PTO/SB/17)
(Submit an original and a duplicate for fee processing)
- ☒ Specification Total Pages **63**
- ☐ Drawing(s) (35 U.S.C. 113) Total Sheets
- ☒ Oath or Declaration Total Pages **3**
- a. ☒ Newly executed (original)
- b. ☐ Copy from a prior application (37 C.F.R. §1.63(d))
(for continuation/divisional with box 15 completed)
- i. ☐ **DELETION OF INVENTOR(S)**
Signed statement attached deleting inventor(s) named
in the prior application, see 37 C.F.R. §1.63(d)(2) and
1.33(b).
5. ☐ Incorporation By Reference (usable if box 4B is checked)
The entire disclosure of the prior application, from which a copy of the
oath or declaration is supplied under Box 4B, is considered to be part
of the disclosure of the accompanying application and is hereby
incorporated by reference therein.

ACCOMPANYING APPLICATION PARTS

6. ☐ Assignment Papers (cover sheet & document(s))
7. ☐ 37 C.F.R. §3.73(b) Statement (when there is an assignee) ☐ Power of Attorney
8. ☐ English Translation Document (if applicable)
9. ☐ Information Disclosure Statement (IDS)/PTO-1449 ☐ Copies of IDS Citations
10. ☐ Preliminary Amendment
11. ☒ White Advance Serial No. Postcard
12. ☐ Small Entity Statement(s) ☐ Statement filed in prior application. Status still proper and desired.
13. ☒ Certified Copy of Priority Document(s) (1)
(if foreign priority is claimed)
14. ☒ Other: Notice of Priority, Receipt of an Original Deposit of Microorganisms for the Purpose of Patent Procedure (FERM BP-6895)

15. If a CONTINUING APPLICATION, check appropriate box, and supply the requisite information below:

☐ Continuation ☐ Divisional ☐ Continuation-in-part (CIP) of prior application no.:

Prior application information: Examiner:

Group Art Unit:

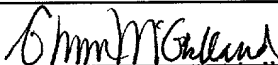
16. Amend the specification by inserting before the first line the sentence:

☐ This application is a ☐ Continuation ☐ Division ☐ Continuation-in-part (CIP)
of application Serial No. Filed on

☐ This application claims priority of provisional application Serial No. Filed

17. CORRESPONDENCE ADDRESS

OBLON, SPIVAK, MCCLELLAND, MAIER & NEUSTADT, P.C.
FOURTH FLOOR
1755 JEFFERSON DAVIS HIGHWAY
ARLINGTON, VIRGINIA 22202
(703) 413-3000
FACSIMILE: (703) 413-2220

Name:	Norman F. Oblon	Registration No.:	24,618
Signature:		Date:	11/16/99
Name:	C. Irvin McClelland	Registration No.:	21,124

METHOD FOR PRODUCING L-METHIONINE BY FERMENTATION

Technical Field

5 The present invention relates to a method for producing L-methionine by fermentation. L-methionine is an important amino acid as a medicament and the like.

Background Art

10

 Industrially produced methionine mainly consists of DL-methionine, which is produced through chemical synthesis. When L-methionine is required, it is provided through production of N-acetyl-DL-methionine by
15 acetylation of DL-methionine and subsequent enzymatic selective deacetylation of the N-acetylated L-methionine.

 On the other hand, as for the production of L-methionine by fermentation, methods utilizing an L-methionine analogue-resistant mutant strain have been
20 reported. However, their production amount is small, and factors affecting the L-methionine production have not been elucidated yet. Therefore, L-methionine is still one of the amino acids the most difficult to be produced by fermentation. For example, while methods
25 utilizing *Escherichia coli* (*E. coli*) K-12 strain have been reported in Japanese Patent Laid-open (Kokai) No. 56-35992 and literature (Chattapadhyay, M.K. et al., Med.

Sci. Res. 23, 775 (1995); Chattopadhyay, M.K. et al., *Biotechnol. Lett.* 17, 567-570 (1995)), any of these methods cannot provide L-methionine production amount sufficient for industrial use.

- 5 In *E. coli*, the biosynthetic pathway of L-methionine is partly shared with the biosynthetic pathway of L-threonine, and L-homoserine serves as a common intermediate. The first step of the peculiar pathway from L-homoserine to L-methionine is catalyzed
- 10 by homoserine transsuccinylase (HTS). This enzyme has been known to suffer concerted inhibition by the final product, L-methionine, and a metabolite of L-methionine, S-adenosylmethionine (Lee, L.-W. et al., *J. Biol. Chem.*, 241, 5479-5480 (1966)).
- 15 The nucleotide sequence of the *metA* gene encoding homoserine transsuccinylase of *E. coli*, has been reported by Duclos et al. (Duclos, B. et al., *Nucleic Acids Res.* 17, 2856 (1989)), and a method for obtaining a strain having a mutation for *metA* using resistance to
- 20 an analog of L-methionine, α -methyl-DL-methionine (MM) has also been known (Chattopadhyay, M.K. et al., *J. Gen. Microbiol.*, 137, 685-691 (1991)). It has been reported for *Salmonella typhimurium* that, the *metA* gene product, homoserine transsuccinylase, was an inhibition-
- 25 desensitized type as for the inhibition by L-methionine and S-adenosylmethionine (SAM) in an MM resistant strain (Lawrence, D.A. et al., *J. Bacteriol.*, 109, 8-11 (1972)).

However, the nucleotide sequence of the mutant *metA* gene has not been reported. Furthermore, it has been reported that a mutant having a sole mutation in *metA* did not secrete L-methionine (Chattopadhyay, M.K. et al.,
 5 *J. Gen. Microbiol.*, 137, 685-691 (1991)).

It has also been revealed that the expression of the genes including *metA* of the enzymes for the reaction by homoserine transsuccinylase and subsequent reactions in the peculiar biosynthetic pathway of L-methionine
 10 suffers inhibition by a repressor which is a *metJ* gene product (Green, R.C. Biosynthesis of Methionine in "*Escherichia coli* and *Salmonella* Cellular and Molecular Biology/Second Edition", ed. Neidhardt, F.D., ASM Press, pp.542-560 (1996)). It has also been known that the
 15 *metJ* gene is adjacent to the *metBL* operon in a reverse direction, which operon consists of the *metB* gene coding for the second enzyme of the peculiar biosynthetic pathway for L-methionine, cystathionine γ -synthase, and *metL* coding for aspartokinase-homoserine dehydrogenase
 20 II (AK-HDII) (Duchange, N. et al., *J. Biol. Chem.*, 258, 14868-14871 (1983)).

It has been suggested that *metK* coding for S-adenosylmethionine, which catalyzes the metabolic reaction from L-methionine to S-adenosylmethionine,
 25 should be an essential enzyme (Green, R.C. Biosynthesis of Methionine in "*Escherichia coli* and *Salmonella* Cellular and Molecular Biology/Second Edition", ed.

Neidhardt, F.D., ASM Press, pp.542-560 (1996)).

Furthermore, it has also been known that a *metJ* mutant strain can be obtained based on resistance to a methionine analogue such as DL-norleucine and ethionine

5 (Chattopadhyay, M.K. et al., *J. Gen. Microbiol.*, 137, 685-691 (1991)), and can increase the expression of the enzymes of the peculiar biosynthetic pathway of L-methionine (Greene, R.C. et al., *J. Bacteriol.*, 115, 57-67).

10 As mentioned above, there have been reported enzymes involved in the L-methionine biosynthesis and genes therefor to some extent. However, only few findings that directly lead to the production of L-methionine by fermentation have been obtained, and hence
15 hardly applied to breeding of L-methionine-producing bacteria.

Summary of the Invention

20 The present invention has been accomplished in view of the aforementioned current technical status, and an object of the present invention is to elucidate factors affecting the L-methionine production, thereby breeding L-methionine-producing bacteria, and enabling
25 L-methionine production by fermentation.

In order to achieve the aforementioned object, the present inventors earnestly conducted studies, and as a

result, accomplished the present invention.

That is, the present invention provides:

- (1) A microorganism which is deficient in repressor of L-methionine biosynthesis system and has L-methionine productivity;
- (2) a microorganism having enhanced intracellular homoserine transsuccinylase activity and L-methionine productivity;
- (3) a microorganism which is deficient in repressor of L-methionine biosynthesis system, and has enhanced intracellular homoserine transsuccinylase activity and L-methionine productivity;
- (4) the microorganism according to any one of the above (1)-(3), which further exhibits reduced intracellular S-adenosylmethionine synthetase activity;
- (5) the microorganism according to any one of the above (2)-(4), wherein the enhanced intracellular homoserine transsuccinylase activity is obtained by increasing copy number of a gene encoding homoserine transsuccinylase, or enhancing an expression regulatory sequence for the gene;
- (6) the microorganism according to the above (1) or (4), which has homoserine transsuccinylase for which concerted inhibition by L-methionine and S-adenosylmethionine is desensitized;
- (7) the microorganism according to any one of the above (1)-(6), which exhibits L-threonine

auxotrophy;

(8) the microorganism according to any one of the above (1)-(7), which exhibits enhanced intracellular cystathionine γ -synthase activity and enhanced
 5 intracellular aspartokinase-homoserine dehydrogenase II activity;

(9) the microorganism according to any one of the above (1)-(8), which belongs to the genus *Escherichia*;

10 (10) a method for producing L-methionine which comprises culturing the microorganism according to any one of the above (1)-(9) in a medium to produce and accumulate L-methionine in the medium, and collecting the L-methionine from the medium; and

15 (11) A DNA which codes for homoserine transsuccinylase for which concerted inhibition by L-methionine and S-adenosylmethionine is desensitized, wherein the homoserine transsuccinylase has the amino acid sequence of SEQ ID NO: 26 including a mutation
 20 corresponding to replacement of arginine by cysteine at the 27th position, mutation corresponding to replacement of isoleucine by serine at the 296th position, mutation corresponding to replacement of proline by leucine at the 298th position, mutation corresponding to
 25 replacement of arginine by cysteine at the 27th position and replacement of isoleucine by serine at the 296th position, mutation corresponding to replacement of

isoleucine by serine at the 296th position and replacement of proline by leucine at the 298th position, mutation corresponding to replacement of proline by leucine at the 298th position and replacement of arginine by cysteine at the 27th position, or mutation corresponding to replacement of arginine by cysteine at the 27th position, replacement of isoleucine by serine at the 296th position and replacement of proline by leucine at the 298th position.

10 In this specification, S-adenosylmethionine will occasionally be abbreviated as "SAM", α -methyl-DL-methionine as "MM", and DL-norleucine as "NL". Further, S-adenosylmethionine synthetase will be occasionally be abbreviated as "SAM synthetase", and homoserine transsuccinylase as "HTS". The *metB* gene product, 15 cystathionine γ -synthase, of *E. coli* may also be called as "cystathionine synthase", and the *metL* gene product, "aspartokinase homoserine dehydrogenase II", may also be called as AK-HDII.

20 The term "L-methionine productivity" used for the present invention means an ability to accumulate L-methionine in a medium when a microorganism is cultured in the medium.

According to the present invention, there is 25 provided a microorganism having L-methionine production ability. The microorganism can be utilized as an L-methionine-producing bacterium or a material for

breeding of L-methionine-producing bacteria.

The mutant *metA* gene of the present invention can be utilized for the breeding of L-methionine-producing bacteria, because the concerted inhibition by L-methionine and SAM for the enzyme encoded by it is canceled.

Detailed Description of the Invention

10 Hereafter, the present invention will be explained in detail.

The microorganism of the present invention is a microorganism which is deficient in repressor of the L-methionine biosynthesis system and has L-methionine productivity, or a microorganism which has enhanced intracellular homoserine transsuccinylase activity and L-methionine productivity. The microorganism of the present invention is preferably a microorganism which is deficient in repressor of the L-methionine biosynthesis system, and has enhanced intracellular homoserine transsuccinylase activity. The microorganism of the present invention further preferably exhibits reduced intracellular SAM synthetase activity.

The aforementioned microorganism of the present invention is not particularly limited, so long as it has a pathway for producing L-methionine and SAM from L-homoserine via O-acylhomoserine which is produced from

L-homoserine by the acyl-transferring reaction, and its expression of the acyl transferase is controlled through suppression by a repressor. While such a microorganism may be an *Escherichia* bacterium, coryneform bacterium, and *Bacillus* bacterium, it is preferably an *Escherichia* bacterium, for example, *E. coli*.

If the microorganism of the present invention is a bacterium in which HTS possessed by the microorganism suffers concerted inhibition by SAM and L-methionine like *E. coli*, its L-methionine productivity may be improved by canceling the inhibition.

As the peculiar pathway for the methionine biosynthesis, there are one which uses cystathionine as an intermediate as in many microorganisms such as *E. coli*, and one which does not use cystathionine as in *Brevibacterium flavum* (Ozaki, H. et al., *J. Biochem.*, 91, 1163 (1982)). In the present invention, a microorganism that uses cystathionine is preferred. In such a microorganism, L-methionine productivity can be increased by enhancing the intracellular cystathionine synthase activity. In addition, even if it is a microorganism like *Brevibacterium flavum*, L-methionine productivity may be enhanced by deficiency of repressor in the L-methionine biosynthesis system and/or enhancement of HTS.

Furthermore, in the aforementioned microorganism, L-methionine productivity can further be increased by

enhancing at least one of the aspartokinase activity and the homoserine dehydrogenase activity, which are involved in the shared pathway of the L-methionine biosynthesis and the L-threonine biosynthesis.

5 When two or more of the aforementioned characteristics are imparted to a microorganism, the order for imparting them is not particularly limited, and they can be given in an arbitrary order. Moreover, when multiple genes are introduced into a microorganism,
10 those genes may be carried by the same vector, or may be separately carried by multiple different vectors. When multiple vectors are used, it is preferred to use vectors having different drug markers and different replication origins.

15 Methods for imparting each of the aforementioned characteristics to a microorganism will be explained below.

20 <1> Deficiency of repressor in L-methionine biosynthesis system

A microorganism deficient in a repressor in the L-methionine biosynthesis system can be obtained by subjecting microorganisms to a mutagenic treatment, and selecting a strain no longer producing the repressor.

25 The mutagenic treatment can be performed with means usually used for obtaining microbial mutants, for example, UV irradiation or treatment with an agent used

for mutagenesis such as N-methyl-N'-nitrosoguanidine (NTG) and nitrous acid.

The repressor can also be made deficient by destroying a gene coding for the repressor on chromosomal DNA of the microorganism. The gene can be destroyed by preparing a deleted type gene which has deletion of at least a part of a coding region or expression regulatory sequence, and causing homologous recombination of the deleted type gene and a gene on the chromosome to substitute the deleted type gene for the gene on the chromosome (gene substitution).

Since the nucleotide sequence of the gene coding for the repressor in the L-methionine biosynthesis system of *E. coli* (*metJ*) has been known (Duchange, N. et al., *J. Biol. Chem.*, 258, 14868-14871 (1983)), the repressor can be isolated from chromosomal DNA, for example, by PCR using primers produced based on the nucleotide sequence. A deleted type gene can be obtained by excising a certain region from the gene fragment obtained as described above, and deleting at least a part of the coding region or expression regulatory region.

The gene substitution can be performed, for example, as follows. A deleted type gene is introduced into a vector having a temperature sensitive replication origin to prepare a recombinant vector, and a microorganism is transformed with the recombinant vector

so that the deleted type gene should be inserted into a gene on chromosomal DNA by homologous recombination of the deleted type gene and the gene on the chromosomal DNA. Then, the transformant strain is cultured at a temperature at which the vector cannot replicate to drop out the vector from cytoplasm. Furthermore, the gene is replaced when one copy of the gene on the chromosome is dropped out with the vector. The occurrence of the desired gene substitution can be confirmed by Southern hybridization analysis of the chromosomal DNA of a strain to be tested for the gene substitution.

As a vector for *E. coli* that has a temperature sensitivity replication origin, for example, the plasmid pMAN997 disclosed in Japanese Patent Application No. 9-194603 and the like can be mentioned. As a vector for coryneform bacteria that has a temperature sensitivity replication origin, for example, the plasmid pHSC4 disclosed in Japanese Patent Laid-open No. 5-7491 and the like can be mentioned. However, the vector is not limited to these, and other vectors can also be used.

As mentioned above, it has known for *E. coli* that the *metJ* gene is adjacent to the *metBL* operon, which consists of *metB* gene and *metL* gene, in the reverse direction (Duchange, N. et al., *J. Biol. Chem.*, 258, 14868-14871 (1983)). Therefore, if a suitable promoter sequence is ligated to a deleted type *metJ* gene and gene substitution is performed as described above, the

destruction of the *metJ* gene and improvement of
 expression utilizing substitution of promoter in the
metBL operon can simultaneously be obtained by one
 homologous recombination. Improved expression of the
 5 *metBL* operon enhances the intracellular cystathionine
 synthase activity and AK-HDII activity.

Specifically, the following three components, a
 fragment of about 1 kb containing the *metB* gene, which
 is obtained by, for example, PCR (polymerase chain
 10 reaction; White, T. J. et al.; *Trends Genet.*, 5, 185
 (1989)) utilizing chromosomal DNA of *E. coli* W3110
 strain as a template, and oligonucleotides having the
 nucleotide sequences of SEQ ID NO: 5 and SEQ ID NO: 6 as
 primers; a fragment of about 1 kb containing the
 15 downstream region of the *metJ* gene obtained by PCR
 utilizing oligonucleotides having the nucleotide
 sequences of SEQ ID NO: 7 and SEQ ID NO: 8 as primers;
 and a sequence having a promoter sequence of the
 threonine operon, which is obtained by annealing of the
 20 oligonucleotides represented as SEQ ID NO: 9 and SEQ ID
 NO: 10, can be inserted into a suitable vector, and
 ligating to it to obtain a recombinant vector which
 contains a DNA fragment having deletion of the
 structural gene of *metJ* and substitution of threonine
 25 promoter for the promoter of the *metBL* operon.

To introduce the recombinant DNA prepared as
 described above to bacterium, any known transformation

methods can be employed. For instance, employable are a method of treating recipient cells with calcium chloride so as to increase the permeability of DNA, which has been reported for *Escherichia coli* K-12 [see Mandel, M. and Higa, A., *J. Mol. Biol.*, 53, 159 (1970)]; and a method of preparing competent cells from cells which are at the growth phase followed by introducing the DNA thereinto, which has been reported for *Bacillus subtilis* [see Duncan, C.H., Wilson, G.A. and Young, F.E., *Gene*, 1, 153 (1977)]. Alternatively, it is also possible to apply a method in which DNA recipient cells are allowed to be in a state of protoplasts or spheroplasts capable of incorporating recombinant DNA with ease to introduce recombinant DNA into the DNA recipient cells, as known for *Bacillus subtilis*, actinomycetes, and yeasts (Chang, S. and Choen, S. N., *Molec. Gen. Genet.*, 168, 111 (1979); Bibb, M. J., Ward, J. M. and Hopwood, O. A., *Nature*, 274, 398 (1978); Hinnen, A., Hicks, J. B. and Fink, G. R., *Proc. Natl. Acad. Sci. USA*, 75, 1929 (1978)). Transformation of coryneform bacteria may be performed by the electric pulse method (refer to Japanese Patent Publication Laid-Open No. 2-207791).

The vector to be used for cloning the genes such as *metA*, *metK* and *thrBC* as described below includes, for example, pUC19, pUC18, pBR322, pHSG299, pHSG399, pHSG398, RSF1010 and the like. Besides, it is possible to use phage DNA vectors. When microorganisms other than *E.*

coli are used, it is preferable to use a shuttle vector autonomously replicable in those microorganisms and *E. coli*. As examples of plasmid autonomously replicable in coryneform bacteria, for example, the followings can be
5 mentioned.

pAM330 (see Japanese Patent Laid-open No. 58-67699)

pHM1519 (see Japanese Patent Laid-open No. 58-77895)

10 pAJ655 (see Japanese Patent Laid-open No. 58-192900)

pAJ611 (see the same)

pAJ1844 (see the same)

15 pCG1 (see Japanese Patent Laid-open No. 57-134500)

pCG2 (see Japanese Patent Laid-open No. 58-35197)

pCG4 (see Japanese Patent Laid-open No. 57-183799)

20 pCG11 (see the same)

pHK4 (see Japanese Patent Laid-open No. 5-7491)

In order to prepare recombinant DNA by ligating the gene fragment and a vector, the vector is digested by restriction enzyme(s) corresponding to the termini of
25 the gene fragment. Ligation is generally performed by using a ligase such as T4 DNA ligase.

The methods to perform, for example, preparation

of the genomic DNA library, hybridization, PCR, preparation of plasmid DNA, digestion and ligation of DNA, and design of oligonucleotide used for primers are described by Sambrook, J., Fritsche, E. F., Maniatis, T. 5 in Molecular Cloning, Cold Spring Harbor Laboratory Press (1989).

<2> Enhancement of HTS activity and introduction of mutant HTS

10 The HTS activity in a microbial cell can be attained by preparing a recombinant DNA through ligation of a gene fragment encoding HTS with a vector which functions in the microorganism, preferably a multi-copy type vector, and transforming the microorganism through 15 introduction of the plasmid into in the microbial cell. As a result of increase of the copy number of the gene encoding HTS in the transformant strain, the HTS activity is enhanced. In *E. coli*, HTS is encoded by the *metaA* gene. When an *Escherichia* bacterium is used as the 20 microorganism, the HTS gene to be introduced is preferably a gene derived from an *Escherichia* bacterium. However, genes derived from other microorganisms having homoserine transacetylase, such as coryneform bacteria, can also be used.

25 Enhancement of HTS activity can also be achieved by introducing multiple copies of the HTS gene into the chromosomal DNA of the above-described host strains. In

order to introduce multiple copies of the HTS gene in the chromosomal DNA of bacterium belonging to the genus *Corynebacterium*, the homologous recombination is carried out using a sequence whose multiple copies exist in the chromosomal DNA as targets. As sequences whose multiple copies exist in the chromosomal DNA, repetitive DNA, inverted repeats exist at the end of a transposable element can be used. Also, as disclosed in Japanese Patent Laid-open No. 2-109985, it is possible to incorporate the HTS gene into transposon, and allow it to be transferred to introduce multiple copies of the HTS gene into the chromosomal DNA. By either method, the number of copies of the HTS gene within cells of the transformant strain increases, and as a result, HTS activity is enhanced.

The enhancement of HTS activity can also be obtained by, besides being based on the aforementioned gene enhancement, enhancing an expression regulatory sequence for the HTS gene. Specifically, it can be attained by replacing an expression regulatory sequence of HTS gene on chromosome DNA or plasmid, such as a promoter, with a stronger one (see Japanese Patent Laid-open No. 1-215280). For example, lac promoter, trc promoter, tac promoter, PR promoter and PL promoter of lambda phage and the like are known as strong promoters. Substitution of these promoters enhances expression of the HTS gene, and hence the HTS activity is enhanced.

Since the nucleotide sequence of the HTS gene (metA) of *E. coli* has been known (Blattner, F.R. et al., *Science*, 277, 1453-1462 (1997)), it can be isolated from chromosomal DNA by PCR using primers produced based on the nucleotide sequence. As such primers, the oligonucleotides having the nucleotide sequences represented as SEQ ID NO: 21 and SEQ ID NO: 22 are specifically mentioned.

It is expected that, by enhancing the HTS activity in a microbial cell as described above, L-methionine biosynthesis can be enhanced, and thus the L-methionine production amount can be increased.

Further, because HTS suffers concerted inhibition by L-methionine and SAM, the L-methionine biosynthesis system can also be enhanced by obtaining a microorganism containing HTS for which concerted inhibition has been canceled. Such a microorganism containing HTS for which concerted inhibition has been canceled can be obtained by subjecting microorganisms to a mutagenic treatment, and selecting a strain containing HTS for which concerted inhibition has been canceled. The mutagenic treatment can be performed with means usually used for obtaining microbial mutants, for example, UV irradiation or treatment with an agent used for mutagenesis such as N-methyl-N'-nitrosoguanidine (NTG) and nitrous acid. The expression "HTS for which concerted inhibition has been canceled" herein used means HTS exhibiting a ratio

of its enzymatic activity in the presence of L-methionine, SAM or L-methionine and SAM to its enzymatic activity in the absence of L-methionine and SAM (remaining ratio) higher than that of a wild-type HTS.

- 5 Specifically, an HTS exhibiting a remaining ratio in the presence of 1 mM L-methionine of 40% or more, preferably 80% or more, a remaining ratio in the presence of 1 mM SAM of 10% or more, preferably 50% or more, or a remaining ratio in the presence of 0.1 mM each of L-
- 10 methionine and SAM of 15% or more, preferably 60% or more is an HTS for which concerted inhibition by L-methionine and SAM has been canceled.

A mutant strain having such a mutant HTS as mentioned above can be obtained by culturing a parent

15 strain in the presence of α -methyl-DL-methionine (MM), e.g., in a medium containing 1 g/l of MM, and selecting a strain growing on the medium. The selection with MM may be repeated two or more times.

A mutant strain having a mutant HTS can also be

20 obtained by cloning the mutant HTS gene (mutant *meta*) from a HTS mutant obtained as described above, and transforming a microorganism with the mutant gene. Isolation of a mutant HTS gene and introduction of the gene into a microorganism can be performed as the

25 aforementioned wild-type HTS gene. As HTS having a mutant *meta* gene, HTS having the amino acid sequence of SEQ ID NO: 26 including a mutation corresponding to

replacement of arginine by cysteine at the 27th position, mutation corresponding to replacement of isoleucine by serine at the 296th position, or mutation corresponding to replacement of proline by leucine at the 298th position can specifically be mentioned. HTS including two or more of these mutations is also a preferred mutant HTS.

<3> Attenuation of SAM synthetase activity

Furthermore, the L-methionine productivity of microorganism can be increased by attenuating intracellular SAM synthetase activity. The L-methionine productivity of a microorganism can also be increased by making the microorganism SAM synthetase activity deficient, but in such a case, the medium for culturing the microorganism must contain SAM. Therefore, it is preferable to attenuate SAM synthetase activity. The expression "attenuating SAM synthetase activity" herein used means that a specific activity of SAM synthetase per unit of protein in microbial cells is made lower than that of a strain having a wild-type SAM synthetase. Specifically, the degree of attenuation, i.e., the reduced activity may be 80% to 50%, preferably 50% to 30%, more preferably 30% to 10% of the SAM synthetase activity of a wild-type strain. In *E. coli*, it has been suggested that, if the specific activity of SAM synthetase falls below 10%, cell division would be

inhibited (Newman, E. B. et al., *J. Bacteriol.*, 180, 3614-3619 (1998)).

The microorganism whose SAM synthetase activity is reduced may be one producing SAM synthetase exhibiting a reduced specific activity per enzymatic protein (reduced type SAM synthetase), or one in which expression efficiency of the enzyme is reduced because of reduced transcription efficiency or reduced translation efficiency of SAM synthetase gene.

10 A mutant strain whose SAM synthetase activity is reduced may be obtained by culturing a parent strain in the presence of DL-norleucine (NL), e.g., in a medium containing 0.1 g/l of NL, and selecting a grown strain. The selection with NL may be repeated two or more times.

15 It is also possible to use ethionine or γ -glutamylmethyl ester instead of DL-norleucine.

A mutant strain having an reduced type SAM synthetase can also be obtained by cloning a gene for the reduced type SAM synthetase from a SAM synthetase-reduced strain obtained as described above, and substituting the mutant gene for a wild-type SAM synthetase gene on a chromosome of microorganism. The gene substitution of SAM synthetase gene can be performed in the same manner as the aforementioned *metJ* gene. Since the nucleotide sequence of the SAM synthetase gene (*metK*) of *E. coli* has been known (Blattner, F.R. et al., *Science*, 277, 1453-1462 (1997)),

20

25

it can be isolated from chromosomal DNA by PCR using primers produced based on the nucleotide sequence. As such primers, the oligonucleotides having the nucleotide sequences represented as SEQ ID NO: 11 and SEQ ID NO: 12 are specifically mentioned. The occurrence of the desired mutation in the obtained *metK* gene can be confirmed by determining the nucleotide sequence of the gene, and comparing it with a known nucleotide sequence of wild-type *metK* gene.

As specific examples of the gene coding for an reduced type SAM synthetase, those coding for SAM synthetases having the amino acid sequence of SEQ ID NO: 18 including a mutation corresponding to replacement of isoleucine by leucine at the 303rd position, mutation corresponding to replacement of valine by glutamic acid at the 185th position, or mutation corresponding to replacement of arginine at the 378th position and subsequent amino acid residues by a sequence of alanine-methionine-leucine-proline-valine (SEQ ID NO: 29) can be mentioned.

<4> L-Threonine auxotrophy

L-methionine productivity can be improved by imparting L-threonine auxotrophy to a microorganism. Specific examples of a microorganism exhibiting L-threonine auxotrophy include those having deficiency of any one of enzymes involved in the peculiar pathway of

L-threonine biosynthesis from L-homoserine to L-threonine. In *E. coli*, the genes of the enzymes involved in the biosynthesis of L-threonine exist as the threonine operon (*thrABC*), and L-threonine auxotrophic strain, which has lost the ability to synthesize L-homoserine and subsequent products, can be obtained by deleting the *thrBC* segment. The *thrA* gene codes for one of the isozymes of aspartokinase, which is an enzyme of the shared pathway of the L-methionine and L-threonine biosyntheses, and hence it is preferably not to be deleted.

In order to delete *thrBC*, the *thrBC* segment in the threonine operon on chromosomal DNA can be destroyed. *thrBC* can be destroyed by replacing the *thrBC* segment on a microbial chromosome with *thrBC* a part of which is deleted. The gene substitution of *thrBC* may be performed in the same manner as in the gene substitution of the aforementioned *metJ* gene. A *thrBC* segment containing deletion can be obtained by amplifying a fragment of about 1 kb containing the upstream region of the *thrB* gene by PCR using *E. coli* chromosomal DNA as a template and primers having the nucleotide sequences of SEQ ID NOS: 1 and 2, similarly amplifying a fragment of about 1 kb containing the downstream region of the *thrC* gene by PCR using primers having the nucleotide sequences of SEQ ID NOS: 3 and 4, and ligating these amplified products.

<5> Production of L-methionine

L-methionine can be produced by culturing a microorganism having L-methionine productivity prepared as described above in a medium so that L-methionine should be produced and accumulated in the medium, and collecting L-methionine from the medium.

The medium to be used may be selected from well-known media conventionally used depending on the kind of microorganism to be used. That is, it may be a usual medium that contains a carbon source, nitrogen source, inorganic ions, and other organic ingredients as required. Any special medium is not needed for the practice of the present invention.

As the carbon source, it is possible to use sugars such as glucose, lactose, galactose, fructose or starch hydrolysate; alcohols such as glycerol or sorbitol; or organic acids such as fumaric acid, citric acid or succinic acid.

As the nitrogen source, it is possible to use inorganic ammonium salts such as ammonium sulfate, ammonium chloride or ammonium phosphate; organic nitrogen such as soybean hydrolysate; ammonia gas; or aqueous ammonia.

It is desirable to allow required substances such as vitamin B1, L-threonine and L-tyrosine or yeast extract to be contained in appropriate amounts as

organic trace nutrients. Other than the above, potassium phosphate, magnesium sulfate, iron ion, manganese ion and the like are added in small amounts, if necessary.

5 Cultivation is preferably carried out under an aerobic condition for 16-120 hours. The cultivation temperature is preferably controlled at 25°C to 45°C, and pH is preferably controlled at 5-8 during cultivation. Inorganic or organic, acidic or alkaline
10 substances as well as ammonia gas or the like can be used for pH adjustment.

Any special techniques are not required for collecting L-methionine from the medium after the cultivation in the present invention. That is, the
15 present invention can be practiced by a combination of well-known techniques such as techniques utilizing ion exchange resin and precipitation.

Best Mode for Carrying out the Invention

20

The present invention will further be explained more specifically with reference to the following examples.

25

Example 1: Acquisition of L-threonine auxotrophic strain and metJ deficient strain from Escherichia coli W3110 strain

<1> Preparation of plasmid for recombination containing *thrBC* structural gene having deletion

Chromosomal DNA was prepared from W3110 strain, which was a derivative of the wild-type K-12 strain of *E. coli*, by using a genomic DNA purification kit (Advanced Genetic Technology) according to the instruction of the kit. Oligonucleotides having the nucleotide sequences of SEQ ID NO: 1 and SEQ ID NO: 2 in Sequence Listing were synthesized. PCR was performed according to the method of Erlich et al. (PCR Technology-Principles and Applications for DNA Amplification, ed. Erlich, H.A., Stockton Press) by using the above oligonucleotides as primers and the aforementioned chromosomal DNA as the template to amplify a fragment of about 1 kb containing the upstream region of the *thrB* gene. This amplification fragment was introduced with recognition sequences for *EcoRI* and *SalI*, which were derived from the primers. The obtained amplified fragment was digested with restriction enzymes corresponding to the introduced recognition sites.

Similarly, PCR was performed by using oligonucleotides having the nucleotide sequences of SEQ ID NO: 3 and SEQ ID NO: 4 as primers to amplify a fragment of about 1 kb containing the downstream region of the *thrC* gene. This amplification fragment was introduced with recognition sequences for *SalI* and

*Hind*III, which were derived from the primers. The
 obtained amplified fragment was digested with
 restriction enzymes corresponding to the introduced
 recognition sites. The aforementioned two amplified
 5 fragments, and pHSG398 (TAKARA SHUZO) digested with
*Eco*RI and *Hind*III were ligated by using a ligation kit
 (TAKARA SHUZO), and *E. coli* JM109 competent cells
 (TAKARA SHUZO) were transformed with the ligation
 product. Plasmids were prepared from the transformants
 10 based on the alkaline method (Boirnboim, H.C. et al.,
Nucleic Acids Res., 7, 1513-1523 (1979)) by using a
 Plasmid Extractor PI-50 (Kurabo Industries, Ltd.). From
 the obtained plasmids, a plasmid in which two fragments
 were inserted in the *Eco*RI and *Hind*III recognition sites
 15 through *Sal*I recognition sites was selected based on the
 lengths of inserted fragments. This plasmid contained
 the upstream and the downstream regions of the
 structural gene of *thrBC*, and contained a gene fragment
 in which substantially full length of the structural
 20 gene of *thrBC* was deleted.

<2> Production of *thrBC* structural gene deletion strain by genetic recombination

The aforementioned plasmid and the plasmid pMAN997
 25 having a temperature sensitive replication origin, which
 was disclosed in Japanese Patent Application No. 9-
 194603, were digested with *Eco*RI and *Hind*III, and

ligated each other. The *E. coli* JM109 strain was transformed with the obtained recombinant plasmid. Plasmids were extracted from the transformants, and one having a structure where a *thrBC*-deleted gene fragment was inserted in pMAN997 was selected, and designated as pMAN Δ BC. The W3110 strain was transformed with this plasmid to perform genetic recombination in a conventional manner. That is, selection of recombinant strains was carried out based on the L-threonine auxotrophy in M9 medium (Sambrook, J. et al., "Molecular Cloning: A Laboratory Manual/Second Edition", Cold Spring Harbor Laboratory Press, A.3 (1989)), and the obtained L-threonine auxotrophic strain was designated as W Δ BC strain.

15

<3> Production of *metJ* deficient strains from W3110 strain and W Δ BC strain

Then, PCR was performed by using the W3110 strain chromosomal DNA as the template and oligonucleotides having the nucleotide sequences of SEQ ID NO: 5 and SEQ ID NO: 6 as primers to amplify a fragment of about 1 kb containing the *metB* gene. This amplification fragment was introduced with recognition sequences for *EcoRI* and *SphI*. The obtained amplified fragment was digested with restriction enzymes corresponding to the introduced recognition sites.

25

Similarly, PCR was performed by using

oligonucleotides having the nucleotide sequences of SEQ ID NO: 7 and SEQ ID NO: 8 as primers to amplify a fragment of about 1 kb containing the downstream region of the *metJ* gene. This amplification fragment was introduced with recognition sequences for *SalI* and *HindIII*. The obtained amplified fragment was digested with restriction enzymes corresponding to the introduced recognition sites.

Then, a sequence represented as SEQ ID NO: 9, which contained *SphI* and *HindIII* recognition sites at the both ends and the promoter sequence of the threonine operon, and its complementary strand represented as SEQ ID NO: 10 were synthesized, annealed, and digested with restriction enzymes *SphI* and *HindIII*. The threonine promoter fragment obtained as described above, pHSG298 (TAKARA SHUZO) digested with *EcoRI*, and the aforementioned two PCR amplification fragments were mixed, and ligated. The JM109 strain was transformed with this ligation solution, and plasmids were extracted from the transformants. A plasmid comprising ligated four of the components was selected from the obtained plasmids. This plasmid had a structure where the *metJ* structural gene was deleted, and the promoter of *metBL* operon was replaced with the threonine promoter.

The plasmid obtained above and the plasmid pMAN997 having a temperature sensitive replication origin, which is disclosed in Japanese Patent Application No. 9-194603,

were digested with *EcoRI*, and ligated each other. A plasmid having a structure where a *metJ*-deleted fragment was inserted into pMAN997 was selected, and designated as pMANΔJ. The W3110 strain and the WΔBC strain were transformed with this plasmid to perform genetic recombination in a conventional manner. Selection of recombinant strains was performed based on the lengths of amplified products from PCR utilizing DNA prepared from the cells as the template, and the oligonucleotides represented in SEQ ID NO: 6 and SEQ ID NO: 8 as primers. The *metJ*-deleted strains obtained from the W3110 strain and the WΔBC strain were designated as WΔJ strain and WΔBCΔJ strain, respectively.

In order to confirm the effect of the *metJ* deletion by the recombination, a crude enzyme extract was prepared from the cells, and the activities of HTS and cystathionine synthase were measured. The W3110 strain and the WΔJ strain were each inoculated to 2 ml of LB medium, and cultured at 37°C overnight. 1 ml of the medium was centrifuged at 5,000 rpm for 10 minutes, and the cells were washed twice with 0.9% saline. The obtained cell were suspended in 1 ml of 0.9% saline, 0.5 ml of which was inoculated to 50 ml of Davis-Mingioli minimal medium (Davis B.D., and Mingioli, E.S., J. Bacteriol., 60, 17-28 (1950)) containing 5 mM L-methionine. The cells were cultured at 37°C for 24 hours, then the medium was centrifuged at 8,000 rpm for

10 minutes, and the cells were washed twice with 0.9% saline. The cells were suspended in 3 ml of 50 mM potassium phosphate buffer (pH 7.5) containing 1 mM dithiothreitol. This suspension was subjected to a cell
5 disruption treatment at 4°C with a power of 150 W for 5 minutes by using an ultrasonicator (Kubota Co.). The sonicated suspension was centrifuged at 15,000 rpm for 30 minutes, and the supernatant was desalted in a Sephadex G-50 column (Pharmacia) to obtain a crude
10 enzyme extract. The HTS activity and the cystathionine synthase activity in the crude enzyme extract were measured.

As for the HTS activity, 5 µl of the crude enzyme extract was added to a reaction mixture comprising 0.1 M
15 potassium phosphate (pH 7.5), 1 mM succinyl-coenzyme A (Sigma), 0.2 nM DL-[¹⁴C]homoserine (Muromachi Chemical Industry), and 0.2 mM L-homoserine to obtain a volume of 50 µl, and allowed to react at 30°C for 10 minutes. 1 µl of the reaction mixture was spotted on a cellulose
20 plate (Merck), and developed with a mixed solvent containing acetone, butanol, water, and diethylamine at a ratio of 10:10:5:2. After the plate was air-dried, autoradiography was performed by using an image analyzer (Fuji Photo Film).

25 The cystathionine synthase has been known to produce α-ketobutyric acid, ammonia, and succinic acid from O-succinylhomoserine in the absence of L-cysteine,

and this can be utilized for simple detection thereof (Holbrook, E.L. et al., *Biochemistry*, 29, 435-442 (1990)). 100 μ l of the crude enzyme extract was added to a reaction mixture comprising 0.2 M Tris-HCl (pH 8), 5 mM O-succinylhomoserine (Sigma), and 0.25 mM pyridoxal phosphate (Sigma) to obtain a volume of 1 ml, allowed to react at 37°C for 20 minutes, and cooled with ice. The O-succinylhomoserine in this reaction mixture was quantitated by reverse phase HPLC (GL Sciences), and the reduced amount of O-succinylhomoserines was calculated by using a reaction mixture not added the crude enzyme extract. The reaction was also performed with no addition of pyridoxal phosphate, and pyridoxal phosphate-dependent reduction of O-succinylhomoserine was defined to be the cystathionine synthase activity.

The specific activities for the HTS activity and the cystathionine synthase activity measured as described above were shown in Table 1. While the HTS activity was hardly detected in the W3110 strain due to the effect of L-methionine addition, marked activity was observed in the WΔJ strain. As also for the cystathionine synthase activity, remarkable increase was observed in the WΔJ strain compared with the W3110 strain. From these results, the effects of the *metJ* deletion and the promoter substitution in the *metBL* operon by recombination were confirmed.

Table 1: HTS activity and cystathionine synthase activity in *metJ* deficient strain

Strain	HTS activity (mmol/min/mg protein)	Cystathionine synthase activity (mmol/min/mg protein)
W3110	0.3	140
WΔJ	126	1300

Example 2: Acquisition of *metK* mutant from W3110 strain

5

The W3110 strain was cultured in LB medium (Sambrook, J. et al., "Molecular Cloning: A Laboratory Manual/Second Edition", Cold Spring Harbor Laboratory Press, A.1 (1989)) at 37°C overnight. 1 ml of the

10 cultured medium was centrifuged at 5,000 rpm for 10 minutes, and the cells were washed twice with 0.9% saline. The obtained cells were suspended in 100 µl of 0.9% saline, 10 µl of which was inoculated to 5 ml of Davis-Mingioli minimal medium containing 0.1 g/l of DL-

15 norleucine (NL), and cultured at 37°C for 5 days.

Some of the grown colonies were subjected to colony separation on LB agar medium, and their growth was confirmed again in Davis-Mingioli minimal medium containing 0.1 g/l of NL to select 12 NL-resistant

20 strains. Chromosomal DNA was prepared from these resistant strains. PCR was performed by using this chromosomal DNA as a template and two sorts of primers having the sequences of SEQ ID NOS: 11 and 12 to amplify the *metK* gene. The nucleotide sequence of this

amplification fragment was determined by using
 amplification primers of which sequences are shown as
 SEQ ID NO: 11 and 12, and primers of which sequences are
 shown as SEQ ID NOS: 13, 14, 15, and 16. The nucleotide
 5 sequence determination was performed by using a Dye
 Terminator Cycle Sequencing Kit (Perkin-Elmer) on a DNA
 sequencer Model 373S (Perkin-Elmer) in accordance with
 the instructions attached to them. The nucleotide
 sequence of the wild strain W3110 determined as a
 10 control completely coincided with the sequence of *metK*
 reported by Blattner et al. (Blattner, F.R. et al.,
Science, 277, 1453-1462 (1997)). This sequence is
 represented as SEQ ID NO: 17. Further, the amino acid
 sequence of SAM synthetase which may be encoded by the
 15 sequence of SEQ ID NO: 17 is shown in SEQ ID NO: 18.

Among the NL resistant strains, a mutation in the
 structural gene of *metK* was found in 3 strains out of
 the 12 strains, which were designated as WNL2, WNL24,
 and WNL32. As for the *metK* nucleotide sequences of
 20 these mutant strains, in the wild-type nucleotide
 sequence shown as SEQ ID NO: 17, the WNL2 strain had
 replacement of adenine by cytosine at the 907th position,
 the WNL24 strain had replacement of thymine by adenine
 at the 554th position, and the WNL32 strain had deletion
 25 of cytosine at the 1132nd position. As a result, it was
 found that, in the amino acid sequence of SAM synthetase
 represented as SEQ ID NO: 18, the SAM synthetase of the

WNL2 strain had replacement of isoleucine by leucine at the 303rd position, that of the WNL24 strain had replacement of valine by glutamic acid at the 185th position, and that of the WNL32 strain had replacement of arginine at the 378th position and subsequent amino acid residues by alanine-methionine-leucine-proline-valine due to deletion of one nucleotide. It was estimated that the SAM synthetase activity was reduced in these strains.

10

Example 3: Production of L-methionine by introduction of *metK* mutation and amplification of wild-type *metA* gene

(1) Introduction of *metK* mutation into WABCAJ strain

15

PCR was performed by using each chromosomal DNA of the *metK* gene mutant strains, WNL2 strain, WNL24 strain, and WNL32 strain, as a template, and oligonucleotides of SEQ ID NO: 19 and SEQ ID NO: 20 as primers to amplify a fragment of about 2.5 kb containing the *metK* gene. This

20

amplification fragment was introduced with recognition sequences for *Hind*III at the both ends. The obtained amplified fragment was digested with *Hind*III. pSTV28

(TAKARA SHUZO) digested with *Hind*III and the PCR amplification fragment were mixed and ligated, and the JM109 strain was transformed with the ligation product. Plasmids were extracted from the transformants. From the obtained plasmids, plasmids inserted with the PCR

25

amplification fragment were selected. As for these plasmids, mutations in the *metK* structural gene were confirmed by determining their nucleotide sequences.

*Hind*III digestion fragments of these plasmids were each cloned into pMAN997 digested with *Hind*III, and the resulting plasmids were designated as pMANK-2, pMANK-24, and pMANK-32, respectively. The W Δ BC Δ J strain was transformed with these plasmids to obtain genetic recombination in a conventional manner. Chromosomal DNA was extracted from the recombinant strains, and used as a template together with oligonucleotides having the nucleotide sequences of SEQ ID NO: 11 and SEQ ID NO: 12 as primers to perform PCR, and the amplification products were examined for nucleotide sequence to select those having mutations. The *metK* mutant strains obtained from the W Δ BC Δ J strain were designated as W Δ BC Δ JK-2 strain, W Δ BC Δ JK-24 strain, and W Δ BC Δ JK-32 strain, respectively.

(2) Amplification of *metaA* gene

PCR was performed by using W3110 strain chromosomal DNA as a template, and oligonucleotides having the nucleotide sequences of SEQ ID NO: 21 and SEQ ID NO: 22 as primers to amplify a fragment of about 1 kb containing the *metaA* gene. This amplification fragment was introduced with recognition sequences for *Sph*I and *Sal*I at the both ends. The obtained amplified fragment

was digested with restriction enzymes corresponding to the introduced recognition sites. The digested product was cloned into pHSG398 digested with *Sph*I and *Sal*I. The nucleotide sequence of the inserted fragment was determined by using amplification primers represented as SEQ ID NOS: 21 and 22, and primers having the sequences of SEQ ID NOS: 23 and 24. The determined nucleotide sequence of *meta*A of the wild strain W3110 completely coincided with the sequence of *meta*A reported by Blattner et al. (Blattner, F.R. et al., *Science*, 277, 1453-1462 (1997)). This sequence is represented as SEQ ID NO: 25. Further, the amino acid sequence of HTS which may be encoded by the sequence of SEQ ID NO: 25 is shown as SEQ ID NO: 26.

15 A *Sph*I and *Sal*I digestion product of this plasmid, *Hind*III and *Sph*I digestion product of the threonine promoter of Example 1, and pMW118 (Nippon Gene) digested with *Hind*III and *Sal*I were mixed and ligated. The JM109 strain was transformed with this reaction mixture, and plasmids were extracted from the transformants. From the obtained plasmids, a plasmid in which the three components were ligated was selected. This plasmid had a structure where the *meta*A gene was positioned at the downstream from the threonine promoter, by which the *meta*A was expressed. This plasmid was designated as pMWPthmeta-W. The W3110 strain, WABC strain, WABCΔJ strain, WABCΔJK-2 strain, WABCΔJK-24 strain, and

W Δ BC Δ JK-32 strain were transformed with this plasmid to obtain transformants.

Each transformant was cultured at 37°C overnight on an LB plate containing 50 mg/l of ampicillin. The
 5 cells were inoculated to 20 ml of medium at pH 7 containing 40 g/l of glucose, 1 g/l of magnesium sulfate, 16 g/l of ammonium sulfate, 1 g/l of potassium dihydrogenphosphate, 2 g/l of yeast extract (Bacto Yeast-Extract, Difco), 0.01 g/l of manganese sulfate,
 10 0.01 g/l of iron sulfate, 30 g/l of calcium carbonate, 50 mg/l of ampicillin, and 0.5 g/l of L-threonine, and cultured at 37°C for 48 hours.

The cells were separated from the culture, and the amount of L-methionine was measured by an amino acid
 15 analyzer (Hitachi). The results are shown in Table 2. L-Methionine, which was not detected for the W3110 strain, was increased in the W Δ BC strain and the W Δ BC Δ J strain. Concerning the *metK* mutant strains, while the amount of L-methionine decreased in the W Δ BC Δ JK-2 strain,
 20 a comparable amount was observed in the W Δ BC Δ JK-32 strain, and the amount was increased in the W Δ BC Δ JK-24 strain. Thus, the effect on the L-methionine production was observed. The W Δ BC Δ JK-24 strain harboring the plasmid pMWPthrmetA-W was given a private number AJ13425,
 25 and it was deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade

and Industry (postal code 305-8566, 1-3 Higashi 1-chome, Tsukuba-shi, Ibaraki-ken, Japan) on May 14, 1998 as an accession number of FERM P-16808, and transferred from the original deposit to international deposit based on
 5 Budapest Treaty on September 27, 1999, and has been deposited as deposition number of FERM BP-6895.

Table 2: L-methionine production amount of wild-type *metA* introduced strains

Strain	Production amount (g/l)
W3110/pMWPthrmetA-W (<i>metA</i> [°])	0.000
WΔBC/pMWPthrmetA-W (<i>thrBC</i> ⁻ , <i>metA</i> [°])	0.008
WΔBCΔJ/pMWPthrmetA-W (<i>metBL</i> [°] , <i>thrBC</i> ⁻ , <i>metA</i> [°])	0.022
WΔBCΔJK-2/pMWPthrmetA-W (<i>thrBC</i> ⁻ , <i>metJ</i> ⁻ , <i>metBL</i> [°] , <i>metK</i> ¹ , <i>metA</i> [°])	0.014
WΔBCΔJK-24/pMWPthrmetA-W (<i>thrBC</i> ⁻ , <i>metJ</i> ⁻ , <i>metBL</i> [°] , <i>metK</i> ¹ , <i>metA</i> [°])	0.141
WΔBCΔJK-32/pMWPthrmetA-W (<i>thrBC</i> ⁻ , <i>metJ</i> ⁻ , <i>metBL</i> [°] , <i>metK</i> ¹ , <i>metA</i> [°])	0.023

10 *metK*¹: reduced *metK*, *metA*[°]: enhanced *metA*,
metBL[°]: enhanced *metBL*

Example 4: Acquisition of *metA* mutant strain and inhibition-desensitized type *metA* gene

15

The W3110 strain was inoculated to 2 ml of LB medium, and cultured at 37°C for 8 hours. 1 ml of the medium was centrifuged at 5,000 rpm for 10 minutes, and the cells were washed twice with 0.9% saline. The
 20 obtained cells were suspended in 100 μl of 0.9% saline,

5 μ l of which was inoculated to 5 ml of Davis-Mingioli minimal medium containing 1 g/l of α -methyl-DL-methionine (MM), and cultured at 37°C for 3 days. The medium was properly diluted, plated on Davis-Mingioli minimal medium containing 1 g/l of MM, and cultured at 37°C overnight. Some of grown colonies were subjected to colony separation on LB agar medium, and their growth was confirmed again on Davis-Mingioli minimal medium containing 1 g/l of MM. This procedure was independently performed 9 times, and six independent resistant strains, each designated as WMM4, WMM5, WMM6, WMM7, WMM8, and WMM9, were obtained.

Chromosomal DNA was prepared from these resistant strains. PCR was performed by using each chromosomal DNA as a template and primers having the sequences represented as SEQ ID NOS: 21 and 22 to amplify the *metaA* gene. The nucleotide sequence of each amplification fragment was determined by using primers for amplification of which nucleotide sequences are represented as SEQ ID NOS: 21 and 22, and primers having the sequences represented as SEQ ID NOS: 23 and 24. As for the *metaA* nucleotide sequence of the resistance strains, in the nucleotide sequence of wild-type *metaA* represented as SEQ ID NO: 25, thymine at the position of 887 was changed to guanine in the WMM4 strain, cytosine at the position of 893 was changed to thymine in the WMM5 strain, and the wild-type sequence was found in the

WMM6 strain, a sequence of ATCTC corresponding the 886th to the 890th nucleotides iterated twice, and an insertion sequence consisting of about 1300 nucleotides, called IS2 (Ghosal, D. et al., *Nucleic Acids Res.*, 6, 1111-1122 (1979)), was present between the repeated sequences in the WMM7 and WMM8 strains, and cytosine at the position of 79 was changed to thymine in the WMM9 strain. As a result, it was found that, in the amino acid sequence of HTS represented as SEQ ID NO: 26, the 296th isoleucine was changed to serine in the WMM4 strain, proline at the position of 298 was changed to leucine in the WMM5 strain, proline at the position of 298 and subsequent amino acid residues were changed to a sequence of arginine-leucine-alanine-proline due to an insertion sequence in the WMM7 and WMM8 strains, and arginine at the position of 27 was changed to cysteine in the WMM9 strain.

The strains WMM4, WMM5, WMM9 and WMM7, in which a mutation was observed in the *metA* structural gene, were each cultured in LB medium contained in a test tube at 37° overnight, and 1 ml of the medium was centrifuged at 5,000 rpm for 10 minutes. The cells were washed twice with 1 ml of 0.9% saline, and suspended in 1 ml of 0.9% saline, 0.5 ml of which was inoculated to 50 ml of minimal medium and cultured at 37°C for one day. The medium was centrifuged at 8,000 rpm for 10 minutes, and the cells were washed twice with 1 ml of 0.9% saline.

The obtained cells were suspended in 3 ml of 50 mM, potassium phosphate buffer (pH 7.5) containing 1 mM dithiothreitol, and a crude enzyme extract was obtained in the same manner as in Example 1. The HTS activity in the crude enzyme extract was measured with the reaction composition mentioned in Example 1 in the presence of an inhibitor. The results are shown in Table 3. The activity was undetectable for the WMM7 strain, and this was considered to reflect the marked decrease of the specific activity due to the amino acid sequence change caused by the insertion sequence. The specific activities of the other strains were about 1/4 of the wild strain. The inhibition by MM was canceled in all of the WMM4, WMM5, and WMM9 strains, and the inhibition by L-methionine was also reduced considerably. While the inhibition by SAM was hardly canceled in the WMM9 strain, tendency of cancellation was observed in the WMM4 and WMM5 strains. Inhibition by the combination of L-methionine and SAM, which exhibited the strongest inhibition for the wild-type HTS activity, was also markedly reduced in the WMM4 and WMM5 strains

Table 3: Activity of HTS derived from MM resistant strains in the presence of various inhibitors

Inhibitor	HTS activity (mmol/min/mg protein)				
	W3110	WMM9	WMM4	WMM5	WMM7
No addition	22.3	5.0	4.5	4.5	0.0
0.1 mM MM	18.6	4.9	4.1	4.6	0.0
1 mM MM	7.0	2.7	4.6	4.8	0.0
0.1 mM Met	14.3	2.5	4.5	4.2	0.0
1 mM Met	0.8	2.2	4.0	4.0	0.0
0.1 mM SAM	17.0	1.1	4.6	3.6	0.0
1 mM SAM	3.0	0.5	2.6	3.3	0.0
0.1 mM SAM + 0.1 mM Met	0.0	0.9	5.6	2.8	0.0

Example 5: L-Methionine production by
introduction of mutant *metA*

5

PCR was performed by using chromosomal DNA from each of the WMM9, WMM4 and WMM5 strains among the *metA* mutants obtained in Example 4 as a template, and

10 oligonucleotides having the sequences of SEQ ID NO: 21 and SEQ ID NO: 22 as primers to amplify a fragment containing the *metA* gene. This amplification fragment had recognition sequences for *SphI* and *SalI* at the both ends. The both ends of this amplified fragment were

15 digested with *SphI* and *SalI*, and cloned into pHSG398 digested with *SphI* and *SalI*. The nucleotide sequence of each insert fragment was determined to confirm the mutation point. A *SphI* and *SalI* digestion product of this plasmid, *HindIII* and *SphI* digestion product of the

20 threonine promoter mentioned in Example 1, and pMW118 (Nippon Gene) digested with *HindIII* and *SalI* were mixed

and ligated. The JM109 strain was transformed with this ligation solution, and plasmids were extracted from the transformants. From the obtained plasmids, those comprising ligated three components were selected, and designated as pMWPthrmetA-9, pMWPthrmetA-4, and pMWPthrmetA-5, respectively.

Further, in order to obtain combination of the mutation points of each mutant *metA* gene, site-specific mutagenesis was performed by using Mutan-Super Express Km (TAKARA SHUZO) according to the instruction of the manufacturer. pMWPthrmetA-9+4 were produced by combining the *metA*-4 mutation with the *metA*-9 mutation using an oligonucleotide having the sequence of SEQ ID NO: 27. pMWPthrmetA-9+5 was similarly produced by combining the *metA*-5 mutation with the *metA*-9 mutation. Furthermore, pMWPthrmetA-9+4+5 was produced by combining the *metA*-9 mutation with the *metA*-4 and *metA*-5 mutations using an oligonucleotide having the sequence of SEQ ID NO: 28.

The WABCAJK-32 strain was transformed with these plasmids to obtain transformants. Each of the transformants was cultured at 37°C overnight on an LB plate containing 50 mg/l of ampicillin. The cells were inoculated to 20 ml of medium at pH 7 containing 40 g/l of glucose, 1 g/l of magnesium sulfate, 16 g/l of ammonium sulfate, 1 g/l of potassium dihydrogenphosphate, 2 g/l of yeast extract (Bacto Yeast-Extract, Difco),

0.01 g/l of manganese sulfate, 0.01 g/l of iron sulfate, 30 g/l of calcium carbonate, 50 mg/l of ampicillin, and 0.5 g/l of L-threonine, and cultured at 37°C for 48 hours. The cells were separated from the culture, and the amount of L-methionine was measured by an amino acid analyzer (Hitachi). The results are shown in Table 4. The amount of L-methionine accumulation increased several times in the strains introduced with a mutant *metA*, compared with the strain introduced with a wild-type *metA*. Furthermore, by combining mutations, further increase of L-methionine production amount was obtained.

Table 4: L-methionine production amount of mutant *metA*-introduced strains

Strain	Amount of L-methionine production (g/l)
WΔBCΔΔJK-32/pMWPthrmetA-W	0.023
WΔBCΔJK-32/pMWPthrmetA-9	0.158
WΔBCΔJK-32/pMWPthrmetA-4	0.108
WΔBCΔJK-32/pMWPthrmetA-5	0.131
WΔBCΔJK-32/pMWPthrmetA-9+4	0.206
WΔBCΔJK-32/pMWPthrmetA-5+9	0.207
WΔBCΔJK-32/pMWPthrmetA-9+4+5	0.236

SEQUENCE LISTING

<110> USUDA, Yoshihiro
KURAHASHI, Osamu

<120> Method for Producing L-Methionine by Fermentation

<130> OP914

<140>

<141> 1999-11-

<150> JP 10-326717

<151> 1998-11-17

<160> 29

<170> PatentIn Ver. 2.0

<210> 1

<211> 28

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:primer

<400> 1

gggaattctg gcaggaggaa ctggcgca

28

<210> 2

<211> 28

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:primer

<400> 2

gggtcgacgc tcatatggc actggaag

28

<210> 3

<211> 28

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:primer

<400> 3

gggtcgacat cagtaaaatc tattcatt

28

<210> 4

<211> 28

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:primer

<400> 4

ggaagcttgc ccgagggaaa gatctgta

28

<210> 5

<211> 28

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:primer

<400> 5

gggcatgcc agggaacttc atcacatg

28

<210> 6

<211> 28

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:primer

<400> 6

gggaattctc atggttcg cgtagag

28

<210> 7

<211> 28

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:primer

<400> 7

ggaagcttgc gtgagatggg gattaacc

28

<210> 8

<211> 28

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:primer

<400> 8

gggaattcta ctgctagctg ctcttgcg

28

<210> 9

<211> 75

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:primer

<400> 9

ggaagcttaa aattttattg acttaggtca ctaaatactt taaccaatat aggcatacg 60
cacagacgca tgccc 75

<210> 10

<211> 75

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:primer

<400> 10

gggcatgcgt ctgtgcgcta tgcctatatt ggtaaagta ttagtgacc taagtcaata 60
aaattttaag cttcc 75

<210> 11

<211> 18

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:primer

<400> 11

caacagtttg agctaacc 18

<210> 12

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:primer

<400> 12

gcggtttttt tgccgatgc 20

<210> 13

<211> 18

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:primer

<400> 13

tcggctacgc aactaatg

18

<210> 14

<211> 18

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:primer

<400> 14

gagaatgcac cgccaccg

18

<210> 15

<211> 18

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:primer

<400> 15

tggcgcgtca cggtagcg

18

<210> 16

<211> 18

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:primer

<400> 16

gcacgtcggg ttcattag

18

<210> 17

<211> 1155

<212> DNA

<213> Escherichia coli

<220>

<221> CDS

<222> (1)..(1152)

<400> 17

atg gca aaa cac ctt ttt acg tcc gag tcc gtc tct gaa ggg cat cct	48
Met Ala Lys His Leu Phe Thr Ser Glu Ser Val Ser Glu Gly His Pro	
1 5 10 15	
gac aaa att gct gac caa att tct gat gcc gtt tta gac gcg atc ctc	96
Asp Lys Ile Ala Asp Gln Ile Ser Asp Ala Val Leu Asp Ala Ile Leu	
20 25 30	
gaa cag gat ccg aaa gca cgc gtt gct tgc gaa acc tac gta aaa acc	144
Glu Gln Asp Pro Lys Ala Arg Val Ala Cys Glu Thr Tyr Val Lys Thr	
35 40 45	
ggc atg gtt tta gtt ggc ggc gaa atc acc acc agc gcc tgg gta gac	192
Gly Met Val Leu Val Gly Gly Glu Ile Thr Thr Ser Ala Trp Val Asp	
50 55 60	
atc gaa gag atc acc cgt aac acc gtt cgc gaa att ggc tat gtg cat	240
Ile Glu Glu Ile Thr Arg Asn Thr Val Arg Glu Ile Gly Tyr Val His	
65 70 75 80	
tcc gac atg ggc ttt gac gct aac tcc tgt gcg gtt ctg agc gct atc	288
Ser Asp Met Gly Phe Asp Ala Asn Ser Cys Ala Val Leu Ser Ala Ile	
85 90 95	
ggc aaa cag tct cct gac atc aac cag ggc gtt gac cgt gcc gat ccg	336
Gly Lys Gln Ser Pro Asp Ile Asn Gln Gly Val Asp Arg Ala Asp Pro	
100 105 110	
ctg gaa cag ggc gcg ggt gac cag ggt ctg atg ttt ggc tac gca act	384
Leu Glu Gln Gly Ala Gly Asp Gln Gly Leu Met Phe Gly Tyr Ala Thr	
115 120 125	
aat gaa acc gac gtg ctg atg cca gca cct atc acc tat gca cac cgt	432
Asn Glu Thr Asp Val Leu Met Pro Ala Pro Ile Thr Tyr Ala His Arg	
130 135 140	
ctg gta cag cgt cag gct gaa gtg cgt aaa aac ggc act ctg ccg tgg	480
Leu Val Gln Arg Gln Ala Glu Val Arg Lys Asn Gly Thr Leu Pro Trp	
145 150 155 160	
ctg cgc ccg gac gcg aaa agc cag gtg act ttt cag tat gac gac ggc	528
Leu Arg Pro Asp Ala Lys Ser Gln Val Thr Phe Gln Tyr Asp Asp Gly	
165 170 175	
aaa atc gtt ggt atc gat gct gtc gtg ctt tcc act cag cac tct gaa	576
Lys Ile Val Gly Ile Asp Ala Val Val Leu Ser Thr Gln His Ser Glu	
180 185 190	
gag atc gac cag aaa tcg ctg caa gaa gcg gta atg gaa gag atc atc	624

Glu Ile Asp Gln Lys Ser Leu Gln Glu Ala Val Met Glu Glu Ile Ile
 -- 195 200 205
 aag cca att ctg ccc gct gaa tgg ctg act tct gcc acc aaa ttc ttc 672
 Lys Pro Ile Leu Pro Ala Glu Trp Leu Thr Ser Ala Thr Lys Phe Phe
 210 215 220
 atc aac ccg acc ggt cgt ttc gtt atc ggt ggc cca atg ggt gac tgc 720
 Ile Asn Pro Thr Gly Arg Phe Val Ile Gly Gly Pro Met Gly Asp Cys
 225 230 235 240
 ggt ctg act ggt cgt aaa att atc gtt gat acc tac ggc ggc atg gcg 768
 Gly Leu Thr Gly Arg Lys Ile Ile Val Asp Thr Tyr Gly Gly Met Ala
 245 250 255
 cgt cac ggt ggc ggt gca ttc tct ggt aaa gat cca tca aaa gtg gac 816
 Arg His Gly Gly Gly Ala Phe Ser Gly Lys Asp Pro Ser Lys Val Asp
 260 265 270
 cgt tcc gca gcc tac gca gca cgt tat gtc gcg aaa aac atc gtt gct 864
 Arg Ser Ala Ala Tyr Ala Ala Arg Tyr Val Ala Lys Asn Ile Val Ala
 275 280 285
 gct ggc ctg gcc gat cgt tgt gaa att cag gtt tcc tac gca atc ggc 912
 Ala Gly Leu Ala Asp Arg Cys Glu Ile Gln Val Ser Tyr Ala Ile Gly
 290 295 300
 gtg gct gaa ccg acc tcc atc atg gta gaa act ttc ggt act gag aaa 960
 Val Ala Glu Pro Thr Ser Ile Met Val Glu Thr Phe Gly Thr Glu Lys
 305 310 315 320
 gtg cct tct gaa caa ctg acc ctg ctg gta cgt gag ttc ttc gac ctg 1008
 Val Pro Ser Glu Gln Leu Thr Leu Leu Val Arg Glu Phe Phe Asp Leu
 325 330 335
 cgc cca tac ggt ctg att cag atg ctg gat ctg ctg cac ccg atc tac 1056
 Arg Pro Tyr Gly Leu Ile Gln Met Leu Asp Leu Leu His Pro Ile Tyr
 340 345 350
 aaa gaa acc gca gca tac ggt cac ttt ggt cgt gaa cat ttc ccg tgg 1104
 Lys Glu Thr Ala Ala Tyr Gly His Phe Gly Arg Glu His Phe Pro Trp
 355 360 365
 gaa aaa acc gac aaa gcg cag ctg ctg cgc gat gct gcc ggt ctg aag 1152
 Glu Lys Thr Asp Lys Ala Gln Leu Leu Arg Asp Ala Ala Gly Leu Lys
 370 375 380
 taa 1155

<210> 18

<211> 384

<212> PRT

<213> Escherichia coli

<400> 18

Met	Ala	Lys	His	Leu	Phe	Thr	Ser	Glu	Ser	Val	Ser	Glu	Gly	His	Pro
1				5				10					15		
Asp	Lys	Ile	Ala	Asp	Gln	Ile	Ser	Asp	Ala	Val	Leu	Asp	Ala	Ile	Leu
			20					25					30		
Glu	Gln	Asp	Pro	Lys	Ala	Arg	Val	Ala	Cys	Glu	Thr	Tyr	Val	Lys	Thr
		35					40					45			
Gly	Met	Val	Leu	Val	Gly	Gly	Glu	Ile	Thr	Thr	Ser	Ala	Trp	Val	Asp
	50					55					60				
Ile	Glu	Glu	Ile	Thr	Arg	Asn	Thr	Val	Arg	Glu	Ile	Gly	Tyr	Val	His
	65				70					75					80
Ser	Asp	Met	Gly	Phe	Asp	Ala	Asn	Ser	Cys	Ala	Val	Leu	Ser	Ala	Ile
				85					90					95	
Gly	Lys	Gln	Ser	Pro	Asp	Ile	Asn	Gln	Gly	Val	Asp	Arg	Ala	Asp	Pro
			100					105					110		
Leu	Glu	Gln	Gly	Ala	Gly	Asp	Gln	Gly	Leu	Met	Phe	Gly	Tyr	Ala	Thr
		115					120					125			
Asn	Glu	Thr	Asp	Val	Leu	Met	Pro	Ala	Pro	Ile	Thr	Tyr	Ala	His	Arg
	130					135					140				
Leu	Val	Gln	Arg	Gln	Ala	Glu	Val	Arg	Lys	Asn	Gly	Thr	Leu	Pro	Trp
	145				150					155				160	
Leu	Arg	Pro	Asp	Ala	Lys	Ser	Gln	Val	Thr	Phe	Gln	Tyr	Asp	Asp	Gly
			165						170					175	
Lys	Ile	Val	Gly	Ile	Asp	Ala	Val	Val	Leu	Ser	Thr	Gln	His	Ser	Glu
		180						185				190			
Glu	Ile	Asp	Gln	Lys	Ser	Leu	Gln	Glu	Ala	Val	Met	Glu	Glu	Ile	Ile
	195					200					205				
Lys	Pro	Ile	Leu	Pro	Ala	Glu	Trp	Leu	Thr	Ser	Ala	Thr	Lys	Phe	Phe
	210					215					220				
Ile	Asn	Pro	Thr	Gly	Arg	Phe	Val	Ile	Gly	Gly	Pro	Met	Gly	Asp	Cys
	225				230					235				240	
Gly	Leu	Thr	Gly	Arg	Lys	Ile	Ile	Val	Asp	Thr	Tyr	Gly	Gly	Met	Ala
			245						250					255	
Arg	His	Gly	Gly	Gly	Ala	Phe	Ser	Gly	Lys	Asp	Pro	Ser	Lys	Val	Asp
		260						265				270			
Arg	Ser	Ala	Ala	Tyr	Ala	Ala	Arg	Tyr	Val	Ala	Lys	Asn	Ile	Val	Ala
		275					280					285			
Ala	Gly	Leu	Ala	Asp	Arg	Cys	Glu	Ile	Gln	Val	Ser	Tyr	Ala	Ile	Gly
	290					295					300				
Val	Ala	Glu	Pro	Thr	Ser	Ile	Met	Val	Glu	Thr	Phe	Gly	Thr	Glu	Lys

305		310		315		320									
Val	Pro	Ser	Glu	Gln	Leu	Thr	Leu	Leu	Val	Arg	Glu	Phe	Phe	Asp	Leu
			325						330					335	
Arg	Pro	Tyr	Gly	Leu	Ile	Gln	Met	Leu	Asp	Leu	Leu	His	Pro	Ile	Tyr
			340					345						350	
Lys	Glu	Thr	Ala	Ala	Tyr	Gly	His	Phe	Gly	Arg	Glu	His	Phe	Pro	Trp
		355				360						365			
Glu	Lys	Thr	Asp	Lys	Ala	Gln	Leu	Leu	Arg	Asp	Ala	Ala	Gly	Leu	Lys
	370					375						380			

<210> 19

<211> 28

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:primer

<400> 19

ggaagcttaa gcagagatgc agagtgcg

28

<210> 20

<211> 28

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:primer

<400> 20

ggaagcttgg tgcgtataa gaggccac

28

<210> 21

<211> 28

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:primer

<400> 21

gggcatgctg tagtgaggta atcaggtt

28

<210> 22

<211> 28

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:primer

<400> 22

gggtcgactt aatccagcgt tggattca

28

<210> 23

<211> 18

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:primer

<400> 23

tgtctgctgg gcggtaca

18

<210> 24

<211> 18

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:primer

<400> 24

agagagtttt tcggtgcg

18

<210> 25

<211> 930

<212> DNA

<213> Escherichia coli

<220>

<221> CDS

<222> (1)..(927)

<400> 25

atg ccg att cgt gtg ccg gac gag cta ccc gcc gtc aat ttc ttg cgt	48
Met Pro Ile Arg Val Pro Asp Glu Leu Pro Ala Val Asn Phe Leu Arg	
1 5 10 15	
gaa gaa aac gtc ttt gtg atg aca act tct cgt gcg tct ggt cag gaa	96
Glu Glu Asn Val Phe Val Met Thr Thr Ser Arg Ala Ser Gly Gln Glu	
20 25 30	
att cgt cca ctt aag gtt ctg atc ctt aac ctg atg ccg aag aag att	144
Ile Arg Pro Leu Lys Val Leu Ile Leu Asn Leu Met Pro Lys Lys Ile	
35 40 45	
gaa act gaa aat cag ttt ctg cgc ctg ctt tca aac tca cct ttg cag	192
Glu Thr Glu Asn Gln Phe Leu Arg Leu Leu Ser Asn Ser Pro Leu Gln	
50 55 60	
gtc gat att cag ctg ttg cgc atc gat tcc cgt gaa tcg cgc aac acg	240
Val Asp Ile Gln Leu Leu Arg Ile Asp Ser Arg Glu Ser Arg Asn Thr	
65 70 75 80	
ccc gca gag cat ctg aac aac ttc tac tgt aac ttt gaa gat att cag	288
Pro Ala Glu His Leu Asn Asn Phe Tyr Cys Asn Phe Glu Asp Ile Gln	
85 90 95	
gat cag aac ttt gac ggt ttg att gta act ggt gcg ccg ctg ggc ctg	336
Asp Gln Asn Phe Asp Gly Leu Ile Val Thr Gly Ala Pro Leu Gly Leu	
100 105 110	
gtg gag ttt aat gat gtc gct tac tgg ccg cag atc aaa cag gtg ctg	384
Val Glu Phe Asn Asp Val Ala Tyr Trp Pro Gln Ile Lys Gln Val Leu	
115 120 125	
gag tgg tcg aaa gat cac gtc acc tcg acg ctg ttt gtc tgc tgg gcg	432
Glu Trp Ser Lys Asp His Val Thr Ser Thr Leu Phe Val Cys Trp Ala	
130 135 140	
gta cag gcc gcg ctc aat atc ctc tac ggc att cct aag caa act cgc	480
Val Gln Ala Ala Leu Asn Ile Leu Tyr Gly Ile Pro Lys Gln Thr Arg	
145 150 155 160	
acc gaa aaa ctc tct ggc gtt tac gag cat cat att ctc cat cct cat	528
Thr Glu Lys Leu Ser Gly Val Tyr Glu His His Ile Leu His Pro His	
165 170 175	
gcg ctt ctg acg cgt ggc ttt gat gat tca ttc ctg gca ccg cat tcg	576
Ala Leu Leu Thr Arg Gly Phe Asp Asp Ser Phe Leu Ala Pro His Ser	
180 185 190	
cgc tat gct gac ttt ccg gca gcg ttg att cgt gat tac acc gat ctg	624

Arg Tyr Ala Asp Phe Pro Ala Ala Leu Ile Arg Asp Tyr Thr Asp Leu
 -- 195 200 205
 gaa att ctg gca gag acg gaa gaa ggg gat gca tat ctg ttt gcc agt 672
 Glu Ile Leu Ala Glu Thr Glu Glu Gly Asp Ala Tyr Leu Phe Ala Ser
 210 215 220
 aaa gat aag cgc att gcc ttt gtg acg ggc cat ccc gaa tat gat gcg 720
 Lys Asp Lys Arg Ile Ala Phe Val Thr Gly His Pro Glu Tyr Asp Ala
 225 230 235 240
 caa acg ctg gcg cag gaa ttt ttc cgc gat gtg gaa gcc gga cta gac 768
 Gln Thr Leu Ala Gln Glu Phe Phe Arg Asp Val Glu Ala Gly Leu Asp
 245 250 255
 ccg gat gta ccg tat aac tat ttc ccg cac aat gat ccg caa aat aca 816
 Pro Asp Val Pro Tyr Asn Tyr Phe Pro His Asn Asp Pro Gln Asn Thr
 260 265 270
 ccg cga gcg agc tgg cgt agt cac ggt aat tta ctg ttt acc aac tgg 864
 Pro Arg Ala Ser Trp Arg Ser His Gly Asn Leu Leu Phe Thr Asn Trp
 275 280 285
 ctc aac tat tac gtc tac cag atc acg cca tac gat cta cgg cac atg 912
 Leu Asn Tyr Tyr Val Tyr Gln Ile Thr Pro Tyr Asp Leu Arg His Met
 290 295 300
 aat cca acg ctg gat taa 930
 Asn Pro Thr Leu Asp
 305

<210> 26

<211> 309

<212> PRT

<213> Escherichia coli

<400> 26

Met Pro Ile Arg Val Pro Asp Glu Leu Pro Ala Val Asn Phe Leu Arg
 1 5 10 15
 Glu Glu Asn Val Phe Val Met Thr Thr Ser Arg Ala Ser Gly Gln Glu
 20 25 30
 Ile Arg Pro Leu Lys Val Leu Ile Leu Asn Leu Met Pro Lys Lys Ile
 35 40 45
 Glu Thr Glu Asn Gln Phe Leu Arg Leu Leu Ser Asn Ser Pro Leu Gln
 50 55 60
 Val Asp Ile Gln Leu Leu Arg Ile Asp Ser Arg Glu Ser Arg Asn Thr
 65 70 75 80
 Pro Ala Glu His Leu Asn Asn Phe Tyr Cys Asn Phe Glu Asp Ile Gln

	85		90		95										
Asp	Gln	Asn	Phe	Asp	Gly	Leu	Ile	Val	Thr	Gly	Ala	Pro	Leu	Gly	Leu
	100				105								110		
Val	Glu	Phe	Asn	Asp	Val	Ala	Tyr	Trp	Pro	Gln	Ile	Lys	Gln	Val	Leu
	115				120								125		
Glu	Trp	Ser	Lys	Asp	His	Val	Thr	Ser	Thr	Leu	Phe	Val	Cys	Trp	Ala
	130				135								140		
Val	Gln	Ala	Ala	Leu	Asn	Ile	Leu	Tyr	Gly	Ile	Pro	Lys	Gln	Thr	Arg
145					150						155				160
Thr	Glu	Lys	Leu	Ser	Gly	Val	Tyr	Glu	His	His	Ile	Leu	His	Pro	His
					165						170				175
Ala	Leu	Leu	Thr	Arg	Gly	Phe	Asp	Asp	Ser	Phe	Leu	Ala	Pro	His	Ser
	180								185						190
Arg	Tyr	Ala	Asp	Phe	Pro	Ala	Ala	Leu	Ile	Arg	Asp	Tyr	Thr	Asp	Leu
	195								200						205
Glu	Ile	Leu	Ala	Glu	Thr	Glu	Glu	Gly	Asp	Ala	Tyr	Leu	Phe	Ala	Ser
	210								215						220
Lys	Asp	Lys	Arg	Ile	Ala	Phe	Val	Thr	Gly	His	Pro	Glu	Tyr	Asp	Ala
225					230						235				240
Gln	Thr	Leu	Ala	Gln	Glu	Phe	Phe	Arg	Asp	Val	Glu	Ala	Gly	Leu	Asp
					245						250				255
Pro	Asp	Val	Pro	Tyr	Asn	Tyr	Phe	Pro	His	Asn	Asp	Pro	Gln	Asn	Thr
					260						265				270
Pro	Arg	Ala	Ser	Trp	Arg	Ser	His	Gly	Asn	Leu	Leu	Phe	Thr	Asn	Trp
	275								280						285
Leu	Asn	Tyr	Tyr	Val	Tyr	Gln	Ile	Thr	Pro	Tyr	Asp	Leu	Arg	His	Met
	290								295						300
Asn	Pro	Thr	Leu	Asp											
305															

<210> 27

<211> 21

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:primer

<400> 27

ccagacgcac aagaagttgt c

<210> 28

<211> 27

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:primer

<400> 28

tagatcgtat agcgtgctct gtagac

27

<210> 29

<211> 309

<212> PRT

<213> Escherichia coli

<400> 29

Ala Met Leu Pro Val

What is claimed is:

1. A microorganism which is deficient in repressor of L-methionine biosynthesis system and has L-methionine productivity.

2. A microorganism having enhanced intracellular homoserine transsuccinylase activity and L-methionine productivity.

3. A microorganism which is deficient in repressor of L-methionine biosynthesis system, and has enhanced intracellular homoserine transsuccinylase activity and L-methionine productivity.

4. The microorganism according to any one of claims 1 to 3, which further exhibits reduced intracellular S-adenosylmethionine synthetase activity.

5. The microorganism according to any one of claims 2 to 4, wherein the enhanced homoserine transsuccinylase activity is obtained by increasing copy number of a gene coding for the intracellular homoserine transsuccinylase, or enhancing an expression regulatory sequence for the gene.

6. The microorganism according to claim 1 or 4,

which has homoserine transsuccinylase for which
concerted inhibition by L-methionine and S-
adenosylmethionine is desensitized.

5 7. The microorganism according to any one of
claims 1 to 6, which exhibits L-threonine auxotrophy.

8. The microorganism according to any one of
claims 1 to 7, which exhibits enhanced intracellular
10 cystathionine γ -synthase activity and enhanced
intracellular aspartokinase-homoserine dehydrogenase II
activity.

9. The microorganism according to any one of
15 claims 1 to 8, which belongs to the genus *Escherichia*.

10. A method for producing L-methionine which
comprises culturing the microorganism according to any
one of claims 1 to 9 in a medium to produce and
20 accumulate L-methionine in the medium, and collecting
the L-arginine from the medium.

11. A DNA which codes for homoserine
transsuccinylase for which concerted inhibition by L-
25 methionine and S-adenosylmethionine is desensitized,
wherein the homoserine transsuccinylase has the amino
acid sequence of SEQ ID NO: 26 including a mutation

corresponding to replacement of arginine by cysteine at the 27th position, mutation corresponding to replacement of isoleucine by serine at the 296th position, mutation corresponding to replacement of proline by leucine at

5 the 298th position, mutation corresponding to replacement of arginine by cysteine at the 27th position and replacement of isoleucine by serine at the 296th position, mutation corresponding to replacement of isoleucine by serine at the 296th position and

10 replacement of proline by leucine at the 298th position, mutation corresponding to replacement of proline by leucine at the 298th position and replacement of arginine by cysteine at the 27th position, or mutation corresponding to replacement of arginine by cysteine at

15 the 27th position, replacement of isoleucine by serine at the 296th position and replacement of proline by leucine at the 298th position.

Abstract

L-Methionine is produced by culturing a microorganism which is deficient in repressor of L-methionine biosynthesis system and/or enhanced intracellular homoserine transsuccinylase activity is cultured in a medium so that L-methionine should be produced and accumulated in the medium, and collecting the L-methionine from the medium. The microorganism preferably further exhibits reduced intracellular S-adenosylmethionine synthetase activity, L-threonine auxotrophy, enhanced intracellular cystathionine γ -synthase activity and enhanced intracellular aspartokinase-homoserine dehydrogenase II activity. The present invention enables breeding of L-methionine-producing bacteria, and L-methionine production by fermentation.

Declaration, Power Of Attorney and Petition

Page 1 of 3

WE (I) the undersigned inventor(s), hereby declare(s) that:

My residence, post office address and citizenship are as stated below next to my name,

We (I) believe that we are (I am) the original, first, and joint (sole) inventor(s) of the subject matter which is claimed and for which a patent is sought on the invention entitled

METHOD FOR PRODUCING L-METHIONINE BY FERMENTATION

the specification of which

☒ is attached hereto.

☐ was filed on _____ as

Application Serial No. _____

and amended on _____.

☐ was filed as PCT international application

Number _____

on _____,

and was amended under PCT Article 19

on _____ (if applicable).

We (I) hereby state that we (I) have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

We (I) acknowledge the duty to disclose information known to be material to the patentability of this application as defined in Section 1.56 of Title 37 Code of Federal Regulations.

We (I) hereby claim foreign priority benefits under 35 U.S.C. § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or § 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or PCT International application having a filing date before that of the application on which priority is claimed. Prior Foreign Application(s)

Application No.	Country	Day/Month/Year	Priority Claimed
10-326717	Japan	17/11/1998	<input checked="" type="checkbox"/> Yes <input type="checkbox"/> No
_____	_____	_____	<input type="checkbox"/> Yes <input type="checkbox"/> No
_____	_____	_____	<input type="checkbox"/> Yes <input type="checkbox"/> No
_____	_____	_____	<input type="checkbox"/> Yes <input type="checkbox"/> No

We (I) hereby claim the benefit under Title 35, United States Code, § 119(e) of any United States provisional application(s) listed below.

_____	_____
(Application Number)	(Filing Date)
_____	_____
(Application Number)	(Filing Date)

We (I) hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s), or § 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. § 112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR § 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application.

Application Serial No.	Filing Date	Status (pending, patented, abandoned)
_____	_____	_____
_____	_____	_____
_____	_____	_____

And we (I) hereby appoint: Norman F. Oblon, Registration Number 24,618; Marvin J. Spivak, Registration Number 24,913; C. Irvin McClelland, Registration Number 21,124; Gregory J. Maier, Registration Number 25,599; Arthur I. Neustadt, Registration Number 24,854; Richard D. Kelly, Registration Number 27,757; James D. Hamilton, Registration Number 28,421; Eckhard H. Kuesters, Registration Number 28,870; Robert T. Pous, Registration Number 29,099; Charles L. Gholz, Registration Number 26,395; Vincent J. Sunderdick, Registration Number 29,004; William E. Beaumont, Registration Number 30,996; Steven B. Kelber, Registration Number 30,073; Robert F. Gnuse, Registration Number 27,295; Jean-Paul Lavalleye, Registration Number 31,451; Timothy R. Schwartz, Registration Number 32,171; Stephen G. Baxter, Registration Number 32,884; Martin M. Zoltick, Registration Number 35,745; Robert W. Hahl, Registration Number 33,893; Richard L. Treanor, Registration Number 36,379; Steven P. Weihrouch, Registration Number 32,829; John T. Goolkasian, Registration Number 26,142; Marc R. Labgold, Registration Number 34,651; William J. Healey, Registration Number 36,160; and Richard L. Chinn, Registration Number 34,305; our (my) attorneys, with full powers of substitution and revocation, to prosecute this application and to transact all business in the Patent Office connected therewith; and we (I) hereby request that all correspondence regarding this application be sent to the firm of OBLON, SPIVAK, McCLELLAND, MAIER & NEUSTADT, P.C., whose Post Office Address is: Fourth Floor, 1755 Jefferson Davis Highway, Arlington, Virginia 22202.

We (I) declare that all statements made herein of our (my) own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Yoshihiro USUDA

NAME OF FIRST SOLE INVENTOR

Yoshihiro Usuda

Signature of Inventor

November 2, 1999

Date

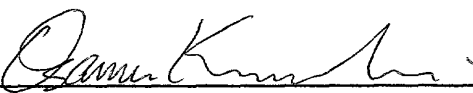
Residence: Kawasaki-shi, Kanagawa, Japan

Citizen of: Japan

Post Office Address: c/o Ajinomoto Co., Inc.,
Fermentation & Biotechnology Laboratories,
1-1, Suzuki-cho, Kawasaki-ku, Kawasaki-shi,
Kanagawa, Japan

Osamu KURAHASHI

NAME OF SECOND JOINT INVENTOR


Signature of Inventor

November 2, 1999

Date

NAME OF THIRD JOINT INVENTOR

Signature of Inventor

Date

NAME OF FOURTH JOINT INVENTOR

Signature of Inventor

Date

NAME OF FIFTH JOINT INVENTOR

Signature of Inventor

Date

Residence: Kawasaki-shi, Kanagawa, Japan

Citizen of: Japan

Post Office Address: c/o Ajinomoto Co., Inc.,
Fermentation & Biotechnology Laboratories,
1-1, Suzuki-cho, Kawasaki-ku, Kawasaki-shi,
Kanagawa, Japan

Residence: _____

Citizen of: _____

Post Office Address: _____

Residence: _____

Citizen of: _____

Post Office Address: _____

Residence: _____

Citizen of: _____

Post Office Address: _____